*Available online at* www.scholarsresearchlibrary.com

Scholars Research Library

**Der Pharmacia Lettre, 2015, 7 (4):129-141**
*(http://scholarsresearchlibrary.com/archive.html)*

Der Pharmacia Lettre
Scholars Research Library
ISSN 0975-5071
USA CODEN: DPLEB4

# Inhibitory effect of exopolysaccharide from *Achromobacter piechaudii* NRC2 against cyclooxygenases and acetylcholinesterase with evaluation of its antioxidant properties and structure elucidation

**Mohsen M. S. Asker[1,*], Manal G. Mahmoud[1], Abeer Y. Ibrahim[2] and Saher S. Mohamed[1]**

[1]*Microbial Biotechnology Department, National Research Centre, 33 Bohouth St., Dokki, Giza, Egypt*
[2]*Medicinal and Aromatic Plants Researches Department, National Research Centre, 33 Bohouth St., Dokki, Giza, Egypt*

---

## ABSTRACT

*Research in Alzheimer's disease (AD) currently includes various cellular, molecular, genetic, clinical and therapeutic approaches. Many epidemiological studies suggest that use of non-steroidal antiinflammatory drugs (NSAIDs) with their antioxidant properties delay or slow the clinical expression of AD. Therefore, in this study we have isolated and characterized both of Achromobacter piechaudii NRC2 by 16S rRNA and its exopolysaccharide (**APEPS**) by HPLC, GPC chromatography and FT-IR spectroscopy. The role of APEPS as cyclooxygenase COX-1 and COX-2 inhibitors (NSAIDs) and as acetyl cholinesterase inhibitor with evaluation of its antioxidative stress effects to mimic AD-like conditions in an in-vitro model at different concentrations was examined. Our results indicated that identified bacterial strain was A. piechaudii NRC2 and APEPS composed of arabinose, xylose, fructose, and galactouronic acid with a relative ratio of 4.5: 4.0: 1.0: 0.3, respectively, with molecular weight of $5.67\times10^3$ g/mol. APEPS showed potent inhibitory effect on COX-2 (inhibition ranged 21 to 92%) and COX-1 (7.77 to 36.22%) as compared to reference tested drug, celecoxib (28 to 100% for COX-2 and 6.11 to 34.12% for COX-1). It also inhibited acetyl cholinesterase concentration dependently, inhibition percentages ranged from 12.36 to 38.35%. These inhibition activities were accompanied with antioxidant effects including radical scavenging effect, $IC_{50}$ were 170.00, 199.31, 205.12 and 73.58 $\mu gmL^{-1}$ for DPPH, superoxide radical, $H_2O_2$ and total antioxidant capacity, respectively. $IC_{50}$ was 100 $\mu gmL^{-1}$ for metal chelation which appeared in inhibition of lipid peroxidation at $IC_{50}$, 112.41 $\mu gmL^{-1}$. These activities may led to slow down or treating Alzheimer disease by different mechanisms.*

**Keywords:** *Achromobacter piechaudii*, Exopolysaccharide, Cyclooxygenase inhibitors, Acetyl cholinesterase inhibitors, Antioxidant.

---

## INTRODUCTION

During the last years, interest in research related to Alzheimer's disease (AD) and inflammation has grown significantly. Ageing is the greatest risk factor for development of AD and this is thought, in part, to be due to enhanced chronic inflammation associated with increasing age [1]. It has been recognized that amyloid-beta (Aβ) is able to initiate an inflammatory response, which implicates the activation of microglia and the recruitment of astrocytes, and therefore the release of cytokines, chemokines, reactive oxygen species and neurotoxic products that have been involved in neuronal and synaptic damage [2]. Over the past decade the process of inflammation has been a focus of increasing interest in the AD field, not only for its potential role in neuronal degeneration but also as a

promising therapeutic target [3]. AD the most common cause of dementia associated with neurodegeneration in the elderly, is characterized clinically by progressive memory loss and other cognitive impairments [4]. Research in AD is rapidly expanding and currently includes various cellular, molecular, genetic, clinical and therapeutic research approaches [5]. The prevalence of AD increases exponentially with age [6]. The free radical hypothesis of aging states that the aging process is associated with multisystem failure due to oxidative damage caused by an imbalance between reactive oxygen species production and antioxidant defenses [7]. Reactive oxygen is a ubiquitous byproduct of both oxidative phosphorylation and the myriad of oxidases necessary to support aerobic metabolism. In AD, there are a number of additional contributory sources that are thought to play important roles in oxidative stress, such as increased neuronal iron in an active redox state, increased nitric oxide (NO) synthesis in microglia, and abnormalities in the mitochondrial genome. Furthermore, lipid peroxidation, a hallmark of oxidative tissue injury, has been found to be elevated in the AD brain [8]. Hence, it is thought that oxidative stress may be an underlying mechanism in AD, and agents that prevent oxidative damage may be particularly efficacious in the treatment of AD [9]. Free radical-mediated lipid per oxidation has been shown to activate cyclooxygenase (COX-2) [10]. Furthermore, the two step oxygenase and peroxidase action of COX leading to the formation of a reactive oxygen species and prostaglandin H2 (PGH2) has been described in detail [11]. However, aggregated synthetic A$\beta$1-40 peptides have been shown to induce COX-2 expression in SH-SY5Y neuroblastoma cells, and A$\beta$1-40 has been shown to stimulate COX-2 oxygenase and peroxidase activity in a cell free system [12]. These findings are further supported by evidence showing that increased basal levels of oxidative stress significantly increases neurotoxicity in hippocampal neurons *in vitro* [13]. Therefore, based on epidemiological studies and experimental work, it is hypothesized that COX-2 plays an important role in neurodegeneration in AD [11]. Many epidemiological studies suggest that use of non-steroidal antiinflammatory drugs (NSAIDs) delay or slow the clinical expression of AD [14-17]. Furthermore, new evidence that COX is involved in neurodegeneration [11, 12, 18] and the development of selective COX inhibitors has led to renewed interest in the therapeutic activity of NSAIDs in AD [19, 20]. AD is characterized by the degeneration of neurological function. The latter is due to the reduction in levels of the neurotransmitter acetylcholine, in the brains of the elderly as the disease progresses, resulting in loss of cognitive ability [21. Acetyl cholinesterase inhibitors (AChEIs) have been shown to function by increasing acetylcholine within the synaptic region, thereby restoring deficient cholinergic neurotransmission [22]. Selective cholinesterase inhibitors, free of dose-limiting side effects, are not currently available, and current compounds may not allow sufficient modulation of acetylcholine levels to elicit the full therapeutic response [21]. In addition, some of the synthetic medicines used *e.g.* tacrine, donepezil and rivastigmine have been reported to cause gastrointestinal disturbances and problems associated with bioavailability [23]. During the past decade varieties of novel exopolysaccharide (EPS) have been obtained from genes *Achromobacter* because it has biological properties [24]. *Achromobacter* is gram negative species produce EPS serve as antitumor [25] because it has no toxicity and causes no side effect have used in many industrial branches and also in medicine [26]. Therefore, the present studies was conduced to isolated and identify both of bacterial strain and its exopolysaccharide as well as evaluate the exopolysaccharide antioxidant properties and inhibitory effect against cyclooxygenase and acetyl cholinesterase enzymes.

## MATERIALS AND METHODS

*Materials*

Ammonium thiocyanate, 1, 3-diethyl thiobarbituric acid (DETBA) and linoleic acid were purchased from Merck. Ferrous chloride, 1,1-diphenyl-2-picryl-hydrazyl (DPPH), 3-(2-pyridyl)-5,6-bis (4-phenyl-sulfonic acid)-1,2,4-triazine (ferrozine), nicotinamide adenine dinucleotide (NADH), butylated hydroxytoluene (BHT), 2,2-azino-bis(3-ethylbenzthiazolin-6-sulphonic acid) diammonium salt (ABTS), peroxidase, potassium hexacyanoferrate, Eserine hemisulfate salt, 5,5-dithiobis-2-nitrobenzoic acid **(**DTNB), $NaH_2PO_4.2H_2O$, $Na_2HPO_4.2H_2O$, ascorbic acid (Vc) and trichloroacetic acid (TCA) were purchased from Sigma-Aldrich (Germany).

*Screening and identification*

Marine sediment samples (3 g) collected from Sidi Bishr (Alexandra, Egypt) were suspended in 90 mL sterile water. Serial dilutions of water samples were plated on marine nutrient agar plates. After incubation at 37°C for 72 h, cultural logogriphic bacterial colony was obtained. Purification of single colonies was done by dilution streaking on marine nutrient agar plates. Single colony cultures were maintained on marine nutrient agar. Pure colonies of each facultative logographic isolates (capable of forming mucoid and ropy colonies) were then inoculated into 50 mL of screening marine nutrient medium in 250-mL conical flask, incubated at 37°C in a rotary shaker at 150 rpm for 48 h. After centrifugation at 4000 rpm for 30 min, the supernatant was mixed with three volumes of chilled ethanol. The

precipitate was collected by centrifugation at 5000 rpm for 30 min and the pellets were dried at 40°C under vacuum. EPS production was determined by quantifying the carbohydrate content of the pellets as glucose equivalents using the phenol-sulfuric acid method [27].

*Biochemical Characterization*
Strain NRC-2, which produces high amounts of EPS, was identified based on biochemical, morphological, and physiological characteristics of the potential producer was determined by adopting standard methods [28, 29].

*Identification of bacteria by 16S rRNA gene sequencing analysis*
*Genomic DNA isolation from isolates*
The isolated bacterial strain was grown in 25 mL broth overnight at 37°C. The culture was centrifuge at 5000 rpm for 7 min. The pellet was re-suspended in 400 µL of sucrose Tris EDTA buffer. Lysozyme was added to a final concentration of 8 mg $mL^{-1}$ and incubated for 1 h at 35°C. To this tube, 100 µL of 0.5 M EDTA (pH 8.0), 60 µL of SDS and 3 µL of proteinase –K (20 mg $mL^{-1}$) were added and incubated at 55°C. After incubation, they were centrifuged at 7000 rpm for 3 min and then the supernatant were extracted twice with phenol: chloroform (1:10, v/v) and again with chloroform: isoamylalcohol (24:1, v/v). It was precipitated with ethanol. The DNA pellet was re-suspended in sterile buffer.

*Amplification of 16S rRNA gene sequence*
A single band of high molecular weight DNA has been observed. A polymerase chain reaction (PCR) was performed using ITS1 (5′-TCCGTAGGTGAACTTTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) primers. Polymerase chain reaction was performed in a typical reaction mixture was 2 µL of template DNA and 1.5 µL of forward primer, 1.5 µL of reverse primer, 10 µL of 2× PCR master mixes and 5 µL of nuclease free water for 20 µL reaction. The reaction was performed with an initial denaturation at 94°C for 2 min 30 cycles of denaturation at 94°C for 45 sec, annealing at 56° for 1 min, extension at 72°C for 1 min followed by final extension at 72°C for 5 min and hold at 4°C. The amplification of 16S rRNA gene was confirmed by running the amplification product in 1% agarose gel electrophoresis. Sequencing was performed by using Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). Sequencing products were resolved on an Applied Bio-systems model 3730XL automated DNA sequencing system (Applied BioSystems, USA). Sequences were submitted to GenBank on the NCBI website (http://www.ncbi.nlm.nih.gov). Sequences obtained in this study were compared to the GenBank database using BLAST software on the NCBI website (http://ncbi.nlm.nih.gov/ BLAST/) [30].

*Production, isolation and purification of polysaccharide*
Inoculum was prepared by transferring one loop full of culture from marine nutrient slant to a 250-mL conical flask containing 50 mL seed medium consisting of (g/L) sucrose 20, peptone 4, yeast extract 2 and dissolved in 750 mL seawater and 250 mL distilled water. The seed cultures were grown at 37 °C on a rotary shaker incubator at 150 rpm for 18 h. After incubation, 3 mL of the seed culture was transferred into a 250-mL conical flask containing 50 mL of production medium consisting of (g/L) sucrose 30, beef extract 1, $(NH_4)_2SO_4$ 0.5, $K_2HPO_4$ $3H_2O$ 2.5, $K_2HPO_4$ 1, NaCl 0.2, $MgSO_4$ $7H_2O$ 0.2, $FeSO_4$ $7H_2O$ 0.001 and 50% sea water [31]. The fermentation cultures were then incubated at 37°C with shaking at 150 rpm for 3 days. The isolate NRC-2 was incubated at 37°C for 96 h. After that, TCA was added to the culture to achieve a final concentration of 5% (w/v), and the mixture was stirred for 30 min at room temperature. Cells and precipitated proteins were removed by centrifugation at 5000 rpm for 25 min at 4°C. The deproteinated solution through precipitation with 1, 2, 3 and 4 volume chilled ethanol and held at 4°C for 24 h. The major pellet was dissolved in deionized water, dialyzed against distilled water using dialysis tubing (MWCO 2000) at 4°C for 24 h, and then lyophilized.

*Monosaccharide composition*
Monosaccharide compositions were determined by high-performance liquid chromatography [32]. The EPS were hydrolyzed with 2 mL (3 M) of trifluoroacetic acid (TFA) at 100°C in a sealed tube for 5 h. Excess acid was removed by flash evaporation on a water bath at a temperature of 40 °C and co-distilled with water [33]. Ethanol was added into the dry sample and evaporated by decompression. The monosugars contents were quantified by HPLC on a Shimadzu Shim-Pack SCR-101N column (7.9 mm × 30 cm), using deionized water as the mobile phase (flow rate 0.5 mL/ min).

*Functional group analysis*
The functional groups of the EPS were detected using Fourier transform infrared (FT-IR) spectroscopy, and the spectrum of the EPS was obtained using a KBr method. The polysaccharide samples were pressed into KBr pellets. The FT-IR spectra were recorded on a Bucker scientific 500-IR Spectrophotometer in the region of 4000–400 $cm^{-1}$ [34].

*Molecular weight determination*
The molecular weight of the EPS was determined by gel permeation chromatography (GPC) on Agilent 1100 series, Germany, Detector: Refractive Index FPl gel particle size (5µm), 3 columns of pore type (100, 104, 105 A°) on series, length 7.5 × 300 mm (1000,5000000) For DMF solvent Styrogel HR-DMF, 3 µm (7.8 × 300 mm), Water Company Ireland. One column (5000-600000) for water solvent (polyethylene oxide/glycol standard) PL aquagel-OH 7.5 mm and 30 um pore type 8 µm particle size. PL aquagel-OH 7.5 mm, 50 µm pore type, 8 µm particle size, in series Mw from 100-1250000 g $mol^{-1}$. The sample 0.01 g was dissolved in 2 mL of solvent, and then it filtrated by siring filter 0.45 then the sample but in GPC device. The polydispersity index calculated from the Mw/Mn ratio [35].

*Antiinflammatory activity*
The cyclooxygenase inhibition assay was performed according to a modified method of Larsen *et al.* [36]. The oxidation of leuco-dichlorofluorescein (L-DCF) in the presence of phenol by the hydroperoxide formed in the cyclooxygenase reaction can be used as a sensitive spectrophotometric assay for PGH synthase activity. Leuco-2, 7-dichloro-fluorescein diacetate (5 mg) was hydrolyzed at room temperature in 1M NaOH (50 µL) for 10 min, then 1M HCl (30 µL) was added to neutralize excess NaOH before the resulting L-DCF was diluted in 0.1 M Tris-buffer, pH 8. COX-1 and COX-2 was diluted in 0.1 M Tris-buffer, pH 8, so that a known aliquot gave an absorbance change of 0.05/min in the test reaction. **APEPS** at 25, 50, 100, 200 and 400 ppm were pre-incubated with enzyme at room temperature for 5 min in the presence of hematin. Premixed phenol, L-DCF and arachidonic acid were added to the enzyme mixture to begin the reaction, and to give a final reaction mixture of arachidonic acid (50 µM), phenol (500 µM), L-DCF (20 µM) and hematin (1 µM) in 1 mL final volume of 0.1 M Tris-buffer, pH 8. The reaction was recorded spectrophotometrically over 1 min at 502 nm. A blank reaction mixture was analyzed in the spectrophotometer reference cell against each test reaction to account for any non-enzymatic activity attributed to the test sample. This blank consisted of the reaction mixture without the addition of enzyme. Celecoxib was used as standard compound.

*Acetyl cholinesterase inhibition assay*
The enzymatic activity was measured using an adaptation of the method described in Ingkaninan *et al.* [37]. 500 µL of DTNB (3 mM), 100 µL of AChI (15 mM), 275 µL of Tris–HCl buffer (50 mM, pH 8 ) and 100 µL of **APEPS** at 25, 50, 100, 200 and 400 µg $mL^{-1}$ were added to a 1 mL and was used as blank. In the reaction, 25 µL of buffer were replaced by the same volume of an enzyme solution containing 0.28 $UmL^{-1}$. The reaction was monitored for 5 min at 405 nm. Velocities of reaction were calculated. Enzyme activity was calculated as a percentage of the velocities compared to that of the assay using buffer instead of inhibitor **APEPS**. Inhibitory activity was calculated from 100 subtracted by the percentage of enzyme activity. Data presented here are the average of three replicates. Eserine hemi sulfate salt was used as positive control and it was tested at different concentration different from samples. The tested eserine concentrations were 0.01, 0.02, 0.04 and 0.08 µg $mL^{-1}$ [38].

Antioxidant properties
*Free radical scavenging activity*
The free radical scavenging activity of **APEPS** was measured using the method of Yamaguchi *et al.* [39]. 0.1 mM solution of DPPH• in methanol was prepared. Then, 1 mL of this solution was added to 3 mL of **APEPS** and standards solution at 25, 50,100, 200 and 400 µg$mL^{-1}$. The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. Then the absorbance was measured at 517 nm using spectrophotometer UV-visible 2401PC (Shimadzu, Japan). Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The $DPPH^{\bullet}$ radical concentration in the reaction medium was calculated from the following equation:

$DPPH^{\bullet}$ scavenging effect (%) = $100 - [(A_0-A_1)/A_0) \times 100]$

Where $A_0$ was the absorbance of the control reaction and $A_1$ was the absorbance in the presence of the sample of polysaccharide.

*Reduction capability*
The method of Oyaizu [40] was used to determine the reducing power effect of **APEPS**. One milliliter of **APEPS** at each different concentration was mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 mL, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 mL) of TCA (10%) was added to the mixture, which was then centrifuged for 10 min at 4200 rpm (MSE Mistral 2000, UK, and Serial No: S693/02/444). The upper layer of solution (2.5 mL) was mixed with methanol (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%), and the absorbance was measured at 700 nm in a spectrophotometer. Ascorbic acid (Vc) and BHT was used as a controls. Higher absorbance of the reaction mixture indicated greater reduction capability.

*Metal chelating effect*
The method of Dinis *et al.* [41] was used to evaluate the chelating effect of **APEPS** and standards against ferrous ions. Briefly, polysaccharide and standards at the same concentrations were added to a solution of 2mM $FeCl_2$ (0.05 mL). The reaction was initiated by the addition of 5mM ferrozine (0.2 mL) and the mixture was shaken vigorously and left standing at room temperature for ten minutes. After the mixture had reached equilibrium, the absorbance of the solution was then measured at 562 nm in a spectrophotometer. The percentage of inhibition of ferrozine-$Fe^{2+}$ complex formation was given by the formula:

$$\text{Inhibition (\%)} = [(A_0-A_1)/ A_0] \times 100$$

Where $A_0$ was the absorbance of the control, and $A_1$ was the absorbance in the presence of the sample of **APEPS** and standards. The control contains $FeCl_2$ and ferrozine.

*Super oxide anion scavenging effect*
Measurement of super oxide anion scavenging activity of **APEPS** was based on the method described by Liu *et al.* [42]. In this experiment, the super oxide radicals were generated in 3ml of Tris-HCl buffer (16mM, pH 8.0) containing 1ml of NBT (50 µM) solution, 1mL NADH (78 µM) solution and 1ml sample solution of **APEPS** at different concentrations were mixed. The reaction was started by adding 1mL of phenazine mehosulphte solution (10 µM) to the mixture and then incubated (25ºC) for 5min. The absorbance at 560 nm in a spectrophotometer was measured against blank samples. Ascorbic acid (Vc) and BHT was used as controls. Decrease in absorbance of the reaction mixture indicated increased super oxide anion scavenging activity. The percentage of inhibition of super oxide anion generation was calculated using the following formula:

$$\text{Inhibition \%} = [(A_0-A_1)/A_0] \times 100$$

Where $A_0$ was the absorbance of the control, and $A_1$ was the absorbance of **APEPS** or standards.

*Scavenging of hydrogen peroxide*
The ability of **APEPS** and standards to scavenge hydrogen peroxide was determined according to the method of Ruch *et al.* [43]. A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). Polysaccharide and standards were added to a hydrogen peroxide solution (0.6 mL, 40 mM). Absorbance of hydrogen peroxide at 230 nm was determined after ten minute against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenging for APEPS and standard compounds was calculated using the following equation:

$$H_2O_2\ (\%) = [(A_0-A_1)\ A_0] \times 100$$

Where $A_0$ was the absorbance of the control, and $A_1$ was the absorbance in the presence of **APEPS** and standards.

*Total antioxidant capacity*
Total antioxidant activity was measured according to the method described by Miller and Rice-Evans [44] and Arnao *et al.* [45]. Exactly, 0.2 mL of peroxidase (4.4 $UmL^{-1}$), 0.2 mL of $H_2O_2$ (50 µM), 0.2 ml of ABTS (100 µM) and 1 mL methanol were mixed, and were kept in the dark for 1hour to form a bluish green complex after adding of 1 mL of **APEPS** at different concentrations or ascorbic acid (Vc) and BHT, all were tested in triplicates. The absorbance at 734 nm was measured to represent the total antioxidant activity and then was calculated as follows:

$$\text{Total antioxidant activity (\%)} = [1- (A_{sample}/A_{control})] \times 100$$

*Lipid per oxidation ammonium thiocyanate*
Inhibition of lipid per oxidation by **APEPS** and standards was determined according to the method of [46]. A pre emulsion was prepared by mixing 175 µg Tween 20, 155 µL linoleic acid, and 0.04 M potassium phosphate buffer (pH 7.0). A 1 mL of sample in 99.5% ethanol was mixed with 4.1 mL linoleic emulsion, 0.02 M phosphate buffer (pH 7.8) and distilled water (pH 7.9). The mixed solutions of all samples were incubated in screw cap-tubes under dark conditions at 40°C at certain time intervals. 0.1 mL of this mixture was pipetted and added with 9.7 mL of 75% and 0.1 mL of 30% ammonium thiocyanate sequentially. After 3 min, 0.1 mL of 0.02 M ferrous chloride in 3.5% HCl was added to the reaction mixture. The peroxide level was determined by reading daily of the absorbance at 500 nm in a spectrophotometer. Ascorbic acid (Vc) and BHT were also determined for comparison. All test data was the average of three replicate. The inhibition of lipid per oxidation in percentage was calculated by the following equation:

$$\text{Inhibition (\%)} = [(A_0 - A_1) / A_0] \times 100$$

Where $A_0$ was the absorbance of the control reaction and $A_1$ was the absorbance in the presence of **APEPS** or standard compounds.

*Statistical analysis*
Analysis of variance (ANOVA) was applied followed with Pot Hoc test to determine differences ($p < 0.01$). Statistical analysis was used to calculate means and standard deviations of three replicates.

## RESULTS AND DISCUSSION

*Screening for bacterial strains producer of EPS*
A wide range of bacteria are known to produce exopolysaccharides. A wide range of biologically active exopolysaccharides are isolated from microorganisms and a number of them are reported to have antitumor and immunomodulatory activities. The mechanisms that mediate the biological activity of polysaccharides are still not clearly understood [47]. Thirty bacterial isolates were recovered from different marine places. A total of thirteen bacterial isolates collected from various marine samples and exhibiting smooth sticky colony and mucoidal morphology on marine agar media were inoculated into shake flasks containing 50 mL of fermentation broth medium. Marine bacterial isolates were screened for their capacity to produce EPS. The highest yield of EPS (9.3 g $L^{-1}$ growth medium) was obtained by a marine bacterium isolated from a Sidi Bishr sample.

*Identification of the strain NRC2*
Identification of bacterial isolate NRC2 was carried out according to morphological, cultural, physiological and biochemical features. The isolated NRC2 had rod shape and was Gram-negative, aerobic, catalase positive, halophilic and capable of growing on different sugars such as glucose, fructose, mannose and arabinose. The organism was able to grow over a wide range of pH (from 5.0 to 10.0). It grew at temperatures ranging from 25 to 45°C wherein the optimal temperature was 37°C. The identification was confirmed by molecular analyses based on 16S rDNA. The 16S rDNA gene of the *Achromobacter* sp. was amplified using polymerase chain reaction (PCR) with the help of 16S rDNA Universal primers. The sequences were compared against 16S rDNA sequences available in the NCBI (http://www.ncbi.nlm.nih.gov) using the BLASTN 2.2.6 program. The sequence analysis revealed that the strains were phylogenetically closely related to the genus *Achromobacter*. Blast analysis of the 16S rDNA sequence of isolate revealed that the selected isolates showed maximum similarity of 98% with *uncultured bacterium* and *Achromobacter sp.* The Phylogenetic relationship was obtained using neighbor joining by pair wise comparison among the 16S rDNA gene sequence of selected isolates with species. The dendrogram was constructed for their Phylogenetic relationship and it revealed that the isolate *Achromobacter piechaudii* NRC2 was distinctly placed under separate clusters. The 16S rRNA gene sequences of the isolates had been submitted to the NCBI Genbank under accession number **KP145014 (Figure 1)**.

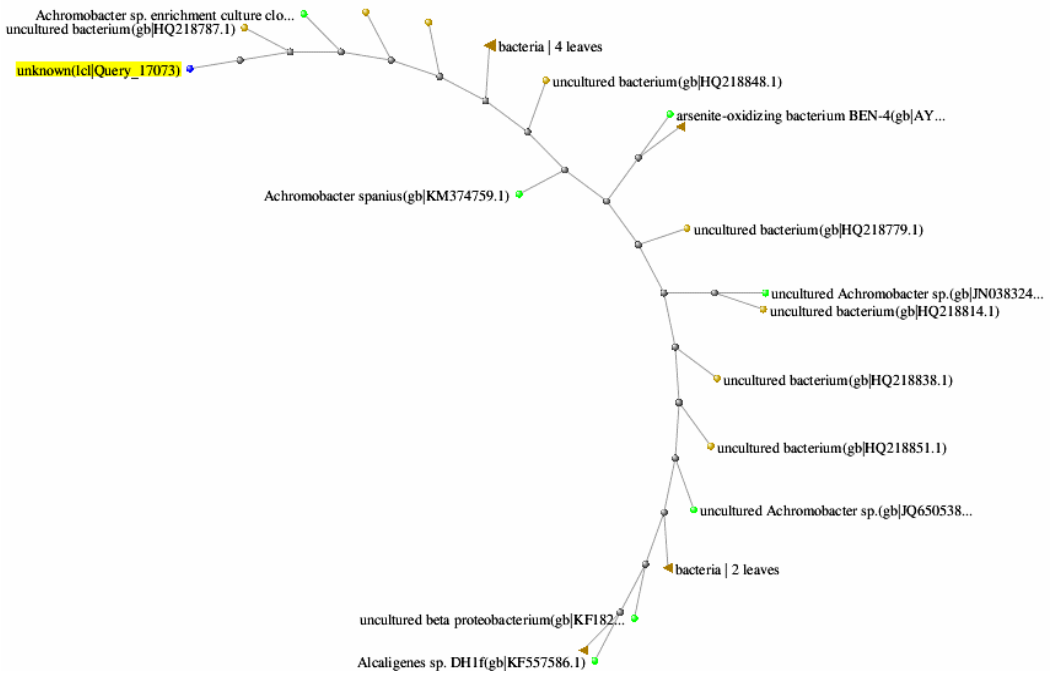


**Figure (1). Phylogenetic tree of the partial sequence of 16S rDNA of the local isolate NRC2 with respect to closely related sequences available in GenBank databases**

*Isolation and chemical structural of APEPS*

Exoolysaccharide production from *A. piechaudii* NRC2 reached a maximum of 9.3 g/L growth medium after 96 h. The main fraction **APEPS** was obtained after fractionation with ethanol precipitation from the crude **EPS**. The **APEPS** was collected for further analysis of structure and biological activity. It appeared as a yellowish powder, with a negative response to the Bradford test. The fact that no absorption was detected by the UV spectra at both 260 and 280 nm indicated the absence of protein and/or nucleic acids. **APEPS** contain uronic acid as evaluated by *m*-hydroxydiphenyl colorimetric method [48] and HPLC analysis. Analysis by HPLC indicated that **APEPS** was composed of arabinose, xylose, fructose, and galactouronic acid with a relative ratio of 4.5: 4.0: 1.0: 0.3, respectively. These results indicated that the presence of arabinogalactan structure within the polysaccharide is important for immunological activity and able to influence inflammatory processes [49]. Also, acidic EPS are useful for protecting against AD and ulcers while, natural EPS lower significant anti-protecting activity [50]. In contrast to the all acidic polysaccharides, pharmacological efficacy of the polysaccharide fractions has not been fully investigated. Nonetheless, several studies have demonstrated that immunostimulatory functions of some acid polysaccharide and there played a critical role in displaying mitogenic, antitumor, and direct immunostimulating activities in cyclophos-phamide-treated immunosuppressed mice [51, 52]. The sugar composition played an important role in the biological activities [53] provided that 101EP and 102EP consisted of arabinose, galactose, glucose, fructose, mannose and maltose stimulated cell proliferation and may be useful as a mild immune modulator of macrophages. The molecular mass value for **APEPS** was calculated for the portions of peaks, which lie within the peak ranges. The weight average molar weight (Mw) of **APEPS** was determined to be $1.64\times10^4$ $gmol^{-1}$ and number average molecular weight (Mn) of $5.67\times10^3$ $gmol^{-1}$. polydispersity index (Mw/Mn) is a measure of the width of molecular weight distribution 2.89 **(Figure 2).**

Kalka-Moll *et al*. [54] reported that the stimulation of cellular immunity dependent on the molecular weight of EPS, their results revealed that, EPS with Mw of 129, 77.8, 46.9 and 17.4 KD stimulated CD4 cell proliferation *in-vitro* at the same time Yan *et al*. [55], showed that EPS-1 derivative for scavenging hydroxyl radical was increased with decreasing molecular weight. Also Deux *et al.* [56], studied the effect of a low Mw fucoidan on SMC proliferation *in-vitro* and intimal hyperplasia *in-vivo*, their results indicated that the low Mw of fucoidan reduced intimal hyperplasia and use for preventional human intent rest enosis. The low values of polydispersity index for all EPS mean that these **EPS** molecules exist much less dispersed in aqueous solution without forming large aggregates [57].

The IR analysis of the **APEPS** is shown in **Figure (3).** There were many peaks from 3430.74 to 616.14 $cm^{-1}$. The band at 3430.74 $cm^{-1}$ region was attributed to the stretching vibration of -OH in the constituent sugar residues [58]. The band at 2967.91 $cm^{-1}$ was associated with the stretching vibration of -CH in the sugar ring. The band at 1647.88 $cm^{-1}$ was due to the stretching vibration of C=O and $COO^-$. The absorptions around 1412.6 $cm^{-1}$ represented -$CH_2$ and -OH bonding. The strong absorption at 1123.33 $cm^{-1}$ was dominated by glycosidic linkage ν(C–O–C)-stretching vibration [59]. Moreover, the band at 921.29 $cm^{-1}$ indicated the β-pyranose form of the glucosyl residue. Therefore, the infrared spectrometry analysis suggested that it was highly likely that the **APEPS** belonged to a β-type hetero-polysaccharide with a pyran group [60].

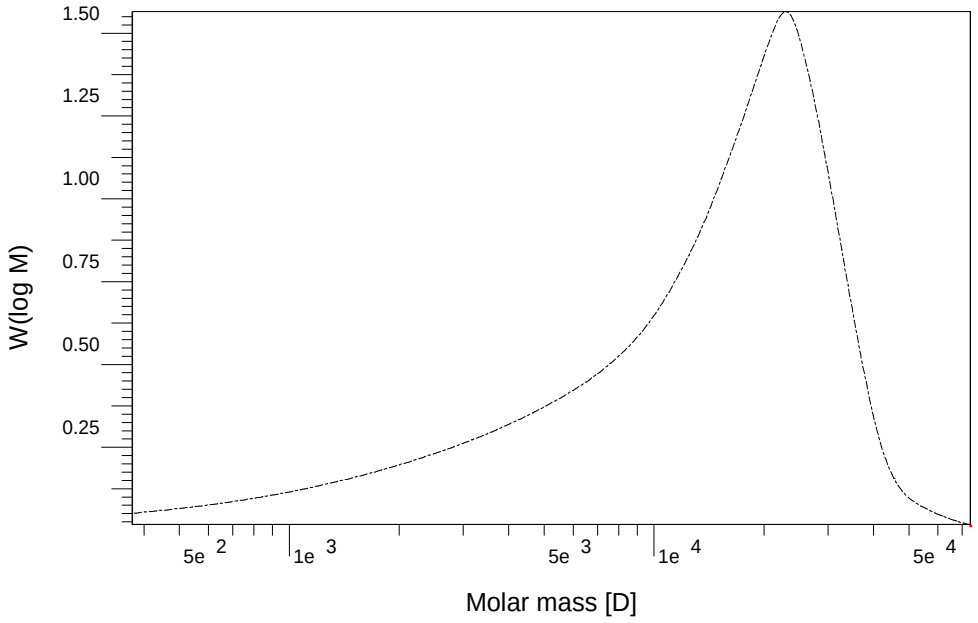


**Figure (2). Molecular weight distributions of APEPS production by *Achromobacter piechaudii* NRC2**

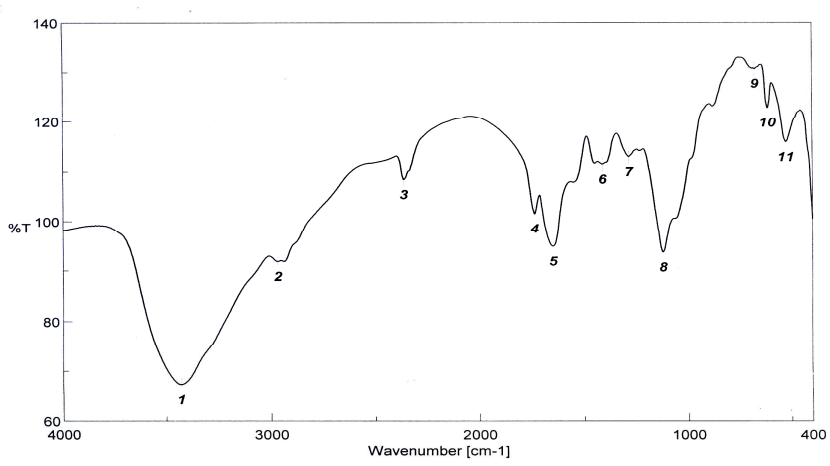


**Figure (3). Infra red spectrum of APEPS from *Achromobacter piechaudii* NRC2**

*Anti-cyclooxygeases activity of APEPS*

The free radical hypothesis of aging states that tissue damage from reactive oxygen species may underlie multisystem failure and these mechanisms may also occur in the progression of AD. Targets of reactive oxygen species include activation of COX-1 and COX-2, which are blocked by NSAIDs. Furthermore, daily doses of

NSAIDs have been shown to increase circulating levels of antioxidants, which combat reactive oxygen species, in many inflammatory related diseases [12]. Therefore, in this study we examined the effectof exopolysaccharide mimics NSAIDs acting on both COX-1 and COX-2 as well as testing if its functional route was supported through its anti-oxidant properties. Free radical-mediated lipid peroxidation has been shown to activate COX-2 [61]. Furthermore, the two step oxygenase and peroxidase action of COX leading to the formation of a reactive oxygen species and prostaglandin H2 (PGH2) [12]. However, aggregated synthetic Amyloid $\beta$ 1-40 (A$\beta$1-40) peptides have been shown to induce COX-2 expression in SH-SY5Y neuroblastoma cells, and A$\beta$1-40 has been shown to stimulate COX-2 oxygenase and peroxidase activity in a cell free system [12]. These findings are further supported by evidence showing that increased basal levels of oxidative stress significantly increase A$\beta$ neurotoxicity in hippocampal neurons [62]. Therefore, based on epidemiological studies and experimental work, it is hypothesized that COX-2 plays an important role in neurodegeneration in AD [12]. Many epidemiological studies suggest that use of non-steroidal anti-inflammatory drugs (NSAIDs) delay or slow the clinical expression of AD [63]. Furthermore, new evidence that COX is involved in neurodegeneration [64] and the development of selective COX inhibitors has led to renewed interest in the therapeutic activity of NSAIDs in AD [19, 20].

In earlier experiments, Nivsarkar *et al.* [65] in a rat model of AD support the hypothesis that COX-2 inhibition alone is an important target for reducing AD-related oxidative stress and recommend that COX-2 inhibitors is most suitable for AD treatment. **APEPS** showed potent anti COX-2 effect as compared to reference tested drug, celecoxib, in a concentration dependent manner. The inhibition percentages ranged from 21% for 25 µg $mL^{-1}$ to 92% for 400 µg $mL^{-1}$, whereas inhibition percentages of celecoxib was ranged from 28% to 100% at the same concentrations **(Figure 4)**. On the other hand, **APEPS** showed plausible effect on COX-1. It exhibited inhibitory effect on COX-1 in a mimic celecoxib manner, the inhibition percentage ranged from 7.77 at 25 µg$mL^{-1}$ to 36.22% at 400 µg $mL^{-1}$ while celecoxibe produced inhibition percentage ranged from 6.11 (25 µg$mL^{-1}$) to 34.12% (400 µg$mL^{-1}$) **(Figure 5)** and there is no significant difference ($P \leq 0.01$) was recorded between celecoxib and APEPS inhibition percentage on COX-1 activity. AD is an irreversible and progressive disorder that leads to an increasingly impaired cognition and eventual death; it is currently treated with palliative, symptomatic therapies [66].

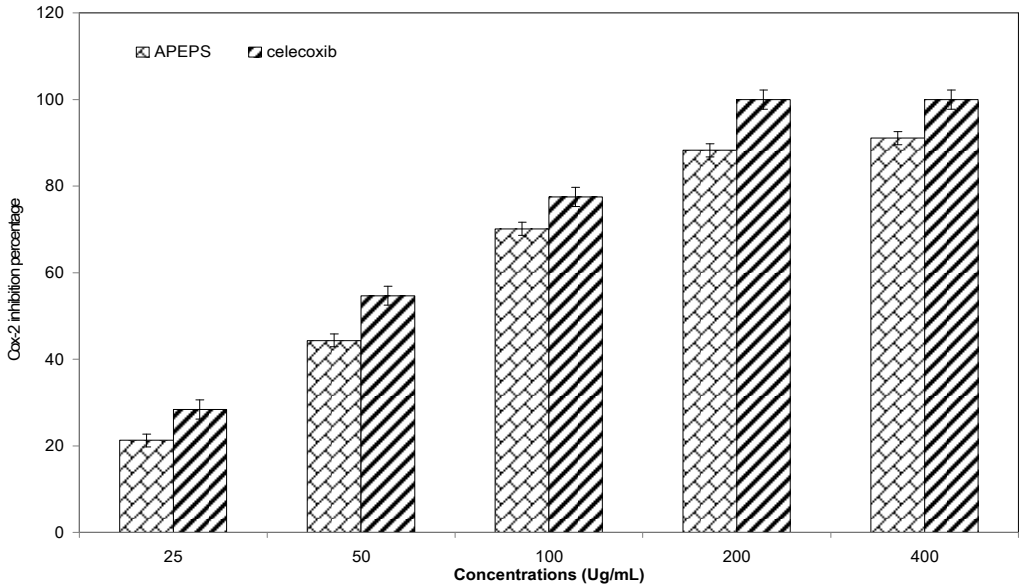


**Figure (4). Inhibition effect of APEPS and celecoxib against COX-2. Data are mean of triplicates ± S.D**

*Anti-acetylcholinesterase activity*

The primary therapeutic strategy, to date, involves the use of cholinesterase inhibitors (ChEIs) to amplify the remaining cholinergic activity. The enzyme, acetyl cholinesterase (AChE), along with other elements of the cholinergic system is depleted in AD. The AChE is a specific cholinesterase, hydrolyzing acetylcholine ester, and found in the brain, nerves and red blood cells (RBC) at a high level [67]. Biochemical studies have indicated that AChE induces amyloid fibril formation by interaction through the peripheral anionic site of the enzyme forming highly toxic Ach E-amyloid-β peptide (Aβ) complexes. The pro-aggregating AChE effect is associated with the intrinsic amyloidogenic properties of the corresponding Aβ peptide. The neurotoxicity induced by AchE-Aβ

complexes is higher than induced by the Aβ peptide alone, both *in vitro* and *in vivo* [19]. Alteration in the level of AChE in situations where in there is a deficiency or absence of ACh assumes significance in clinical conditions such as AD. It was hoped that restoring the cholinergic balance by inhibition of ACh breakdown would slow down the progression of AD and improve cognitive and general functioning [68]. The **APEPX** was evaluated for its inhibitory effect against acetyl cholinesterase in an *in-vitro* model. It inhibited acetyl cholinesterase concentration dependently. The lowest inhibition value was 12.36% at **APEPS** minimum concentration, 25 µgmL$^{-1}$, whereas the inhibitoriest effect was 38.35% at 400 µgmL$^{-1}$ **(Figure 6).** The inhibition percentage was increased significantly ($P \leq 0.01$) with increasing the **APEPS** concentration up to 200 µgmL$^{-1}$. On the other hand, the standard material, eserine hemisulfate, represented minimum recorded $IC_{50}$ was 0.03 µgmL$^{-1}$.

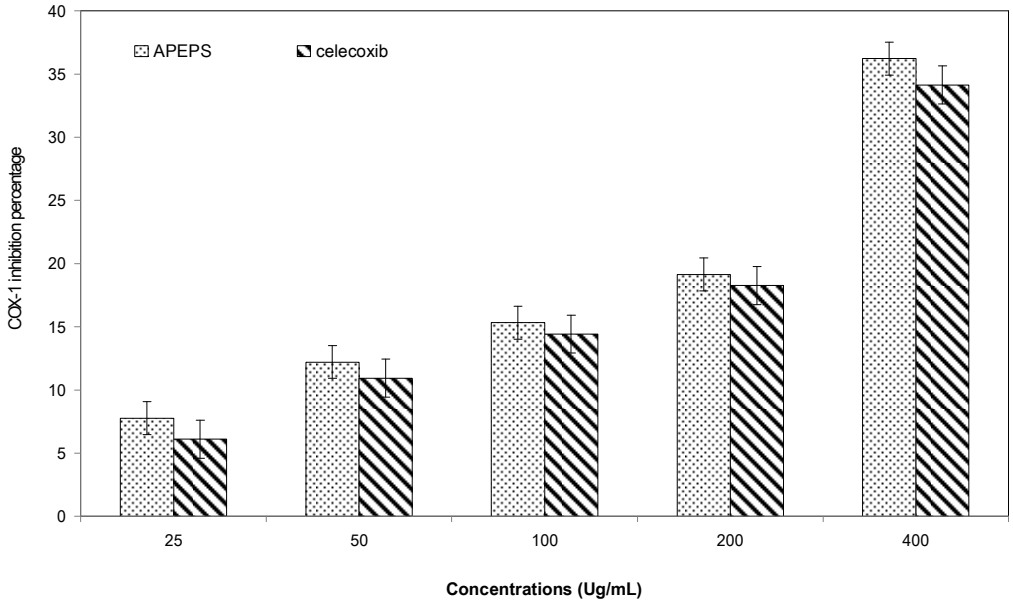


**Figure (5). Inhibition effect of APEPS and celecoxib against COX-1. Data are mean of triplicates ± S.D**

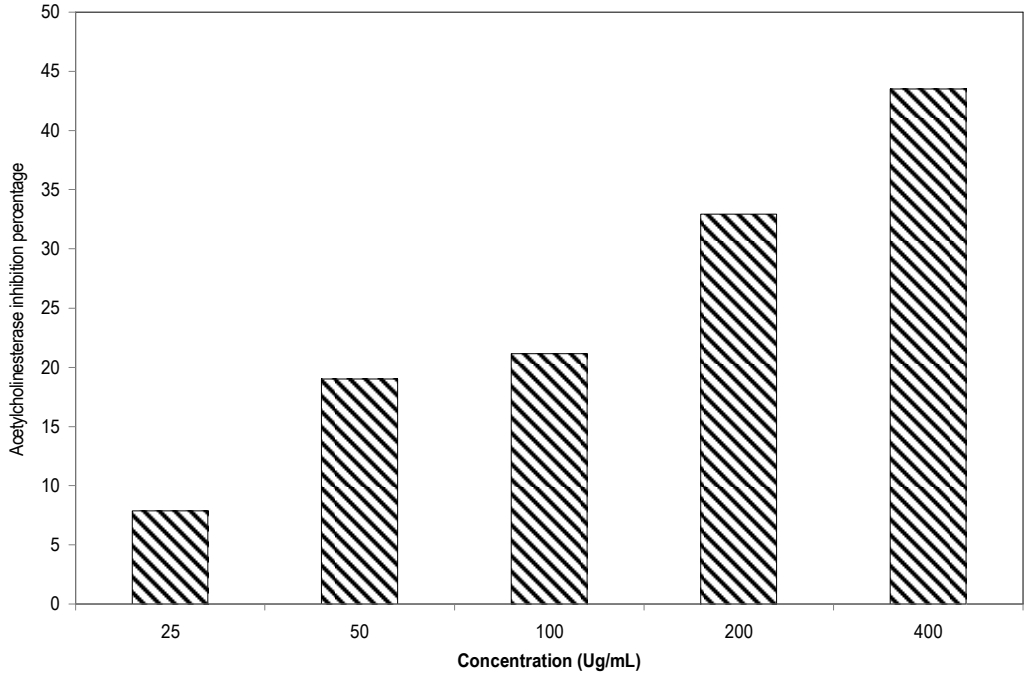


**Figure (6). Acetyl cholinesterase inhibitory effect of APEPS**

*Antioxidant properties of APEPS*

In AD, there are a number of additional contributory sources that are thought to play important roles in oxidative stress, such as increased neuronal iron in an active redox state, increased nitric oxide (NO) synthesis in microglia, and abnormalities in the mitochondrial genome. Furthermore, lipid per oxidation, a hallmark of oxidative tissue injury, has been found to be elevated in the AD brain. Hence it is thought that oxidative stress may be an underlying mechanism in AD, and agents that prevent oxidative damage may be particularly efficacious in the treatment of AD [69].

The antioxidant properties of **APEPS** was evaluated by different assays to be aware of its benefits in scavenging of different types of radicals and chelation of ion metals as well as evaluation of its reduction capability which reflected on its total antioxidant capacity and lipid per oxidation. **APEPS** scavenged DPPH radicals at different concentrations but it remained weak as compared to Vc and BHT $IC_{50}$ was 170, 17.29 and 16.25 $\mu gmL^{-1}$ for APEPS, Vc and BHT, respectively **(Table 1)**. APEPS showed scavenging effect against super oxide radical and hydrogen peroxide radical $IC_{50}$ was 199.31 and 205.12 $\mu gmL^{-1}$ respectively, and its effect on their radicals remained less than its effect against DPPH radicals. It also reproduces a metal chelation effect when it tested on ferrous ion $IC_{50}$ was 100.8 $\mu gmL^{-1}$) which is lower than Vc activity by 48% and BHT by 57%. The ability of **APEPS** to scavenged mentioned radicals as well as chelating effect reflected on its total antioxidant capacity which represented $IC_{50}$ at 73.58 $\mu gmL^{-1}$ and inhibited lipid per oxidation in linoleic acid assay and its $IC_{50}$ was 112.41 $\mu gmL^{-1}$. The reduction capability of APEPS was determined in potassium ferricyanide/ $FeCl_3$ assay. APEPS appeared to have ability to reduce the potassium ferricyanide in a concentration dependent manner; the reduction was increased with increasing concentration of **APEPS** except with increasing **APEPS** from 100 $\mu gmL^{-1}$ to 200 $\mu gmL^{-1}$ which didn't produce any significant effect while the activity was magnified when the concentration reach 400 $\mu gmL^{-1}$ **(Figure 7).**

**Table (1): Antioxidant properties of APEPS and standards (Vc and BHT)**

| | $IC_{50}$ (µg/ml) | | | | | |
|---|---|---|---|---|---|---|
| | DPPH scavenging | $O_2^-$ scavenging | $H_2O_2$ scavenging | Metal chelation | TCA | Lipid per oxidation |
| APEPS | 170±1.01 | 199.31±0.88 | 205.12±1.21 | 100.80±0.89 | 73.58±1.41 | 112.41±2.13 |
| Vc | 17.29±1.05[a] | 51.81±0.91 | 15.17±0.95[b] | 68.76±0.94 | 30.48±1.23[c] | 12.34±1.14[d] |
| BHT | 16.25±0.09[a] | 48.69±0.87 | 15.59±1.41[b] | 57.71±0.1.10 | 28.35±0.98c | 13.25±2.02[d] |

*Data are presented as mean of triplicates ± S.D. The same letter means insignificant* ***difference between these groups***

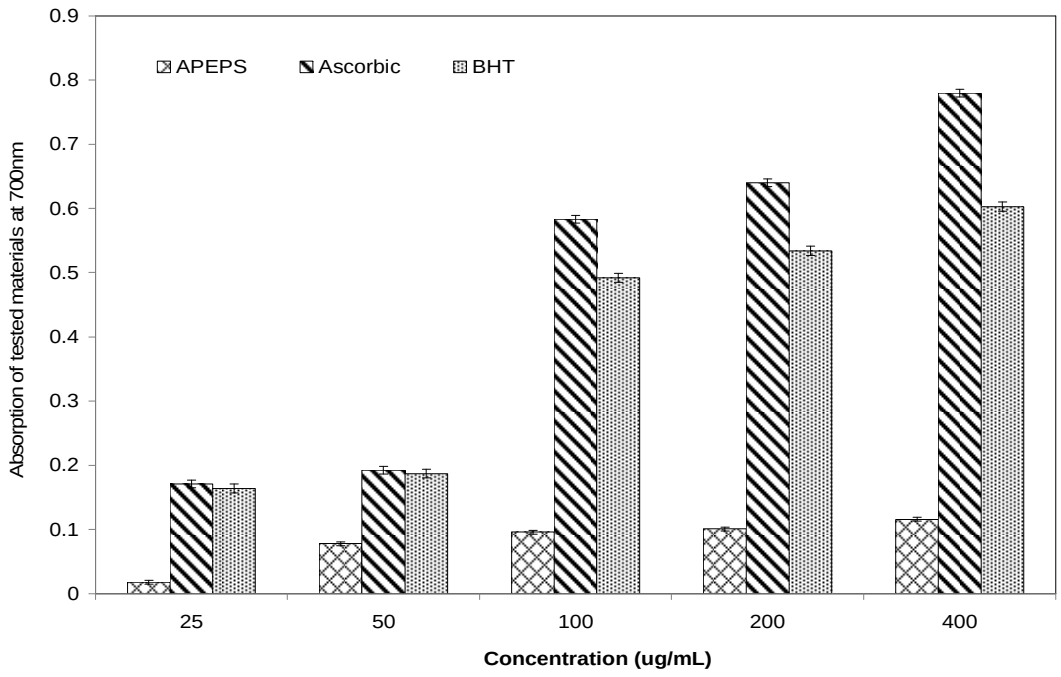


**Figure (7). Reduction capability of APEPS in potassium ferricyanide/$FeCl_3$ system**

As indicated in the literature, acidic polysaccharides are likely to contain more uronic acids with negative charges [70]. The atoms of polysaccharides with a greater proportion of uronic acid were negatively charged, resulting in less steric hindrance when a superoxide anion radical ($O2^{-\bullet}$) attacks. This explains the reason for high content of uronic acid in acidic polysaccharides having higher radical scavenging activities, especially for superoxide anion radicals [70]. Many studies also suggest that the bioactivities of polysaccharides are closely associated with their structures, such as the type of glycosyl units, the configuration of glycosidic bonds, and the substituent of the polysaccharides. In addition, the spatial structure and molecular weight of polysaccharides could also affect the bioactivity [71]. Therefore, the antioxidative activity of the **APEPS** not may be the result of a single factor. It is the result of many factors when combined in the variation of monosaccharide composition, structure configuration, and mode of attending glycosidic bonds, molecular weight and other structural characteristics of the **APEPS** [72]. Regarding the effect of **APEPS** as selective anti-cycloxygenases and its inhibitory effect on acetyl cholinesterase as well as its antioxidant properties, it may a useful natural source in treating and/or controlling Alzheimer disease.

## REFERENCES

[1] Blasko I, Stampfer-Kountchev M, Robatscher P, Veerhuis R, Eikelenboom P, Grubeck-Loebenstein B. *Aging. Cell*, **2004,** 3, 169–176.
[2] Sastre M, Klockgether T, Heneka MT. *Int. J. Dev. Neurosci.*; **2006**, 24,167–176.
[3] Birch AM, Katsouri L, Sastre M. *J. Neuroinflammation*, **2014**, 3, 11:25.
[4] Ritchie K, Lovestone S. *Lancet*, **2002,** 360, 1759–1766.
[5] Lahiri DK, Farlow MR, Greig NH, Sambamurti K. *Drug Dev Res*, **2002**, 56, 267–281.
[6] Katzman R. *N Engl J. Med.*, **1986**, 314, 964–973.
[7] Harman D. *J. Gerontol.*, ***1956,*** 11, 298–300.
[8] Subbarao KV, Richardson JS, Ang LC *J. Neurochem.*, **1990**, 55, 342–345.
[9] Smith MA, Hirai K, Nunomura A, Perry G. *Drug Dev Res*, **1999**, 46, 26–33.
[10] Halliwell B, Gutteridge JMC. Free Radicals in Biology and Medicine. 2ed Oxford. Clarendon Press, **1989,** 422–38. 52.
[11] Pasinetti GM, Aisen PS. *Neuroscience*, **1998,** 87, 319–324.
[12] Pasinetti GM. *J. Neurosci Res*, **1998**, 54, 1–6.
[13] Pike CJ, Ramezan-Arab N, Cotman CW. *J. Neurochem*, **1997**, 69, 1601–1611.
[14] Lucca U, Tettamanti M, Forloni G, Spagnoli A. *Biol. Psychiatry*, **1994,** 36, 854–856.
[15] Andersen K, Launer LJ, Ott A, Hoes AW, Breteler NMB, Hofman A. *Neurology*, **1995**, 45, 1441–1445.
[16] McGeer PL, Schulzer M, McGeer EG. *Neurology*, **1996,** 47, 425–432.
[17] Rich JB, Rasmusson DX, Folstein MF, Carson KA, Kawas C, Brandt J. *Neurology*, **1995**, 45, 51–55.
[18] O'Banion MK. *Expert Opin Investig Drugs*, **1999,** 8, 1521–1536.
[19] Aisen PS. *J. Pain Symptom Manage*, **2002**, 23, 35–40.
[20] Süleyman H, Demircan B, Karagöz Y. *Pharmacol. Rep.*, **2007**, 59 247–258.
[21] Felder CC, Bymaster FP, Ward J, DeLapp N. *J. of Medical Chemistry*, **2000,** 43, 4333 -4353.
[22] Krall WJ, Sramek JJ, Cutler NR. *The Ann. of Pharmacotherapy*, **1999**, 33, 441–450.
[23] Schulz V. *Phytomedicine*, **2003**, 10, 74–79.
[24] Smith JE. J. Biol. Chem., **1968**, 243, 5139-5144.
[25] Watnick PI. The bacterial biofilm matrix as a platform for protein delivery patent **2014**, no. WO 2014008406 A2
[26] Konicek J, Lasik J, Safar H. *Folia Microbiol.*, **1993,** 38, 68–70.
[27] Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. *Ana. Chemistry*, **1956**, 28, 350–356.
[28] Cappuccino JG, Sherman N, **2004**. Microbiology: A Laboratory Manual, Pearson Education (Singapore), Indian Branch. New Delhi.
[29] Collins NM, **1989**. Termites. In: Lieth, H., Werger, M.J.A. (eds). Tropical Rain Forest Ecosystems. Elsevier Science Publishers, Amsterdam; pp. 455-471.
[30] Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. *Molecular Biology and Evolution* **2011**, 28, 2731-2739.
[31] Liu C-F, Tseng K-C, Chiang S-S, Lee B-H, Hsu W-H and Pan T-M. *J. Sci. Food Agric*., **2010**, 91, 2284-2291
[32] El-Sayed OH, Ismail SA, Ahmed YM, Abd El-Samei M, Asker MMS. *Egyptian Pharmaceutical J.*, **2005**, 4, 439–452.
[33] Asker MMS, Ahmed YM, Ramadan MF. *Carbohydrate Polymers* 77: 563–567
[34] Ray B (**2006**). *Carbohydrate Polymers*, **2009**, 66, 408–416.

[35] You L, Qing G, Mengying F, Bao Y, Jiaoyan R, Longjian G, Chun C, Mouming Z. *Food Chemistry* **2013**, 138, 2242–2249
[36] Larsen LN, Dahl E, Bremer J. *Biochim. Biophys. Acta* **1996**, 1299, 47–53.
37- Ingkaninan K, Temkitthawon P, Chuenchon K, Yuyaem T, Thongnoi W. *J. of Ethnopharmacology* **2003**, 89, 261-264.
[38] McPhee S, Hodges LD, Wright PFA, Wynne PM, Kalafatis N, Harney DW, Macrides TA. *Com. Biochemistry and Physiology, Part B,* **2007**, 146, 346- 356.
[39] Yamaguchi T, Takamura H, Matoba T, Terao J. *Biosci. Biotech. Biochem.*, **1998**, 62,1201–1204.
[40] Oyaizu M. *Jpn J Nutr*. **1986**, 44, 307 – 315.
[41] Dinis TCP, Madeira VMC, Almeida LM. *Arch Biochem Biophys.* **1994**, 315, 161 – 169.
[42] Liu F, Ooi VE, Chang ST. *Life Sci.*, **1997**, 60, 763-71.
[43] Ruch RJ, Cheng SJ, Klaunig JF. *Carcinogenesis* **1989**, 10, 1003–1008.
[44] Miller NJ, Rice-Evans CA. *Food Chemistry*, **1997**, 60, 331–337.
[45] Arnao MB, Cano A., Acosta M. *Food Chemistry*, **2001**, 73, 239–244.
[46] Gülçin I, Oktay M, Küfrevio˘glu Ö˙I,Aslan A. Ach. J. of Ethnopharmacology **2002**, 79, 325–329.
[47] Manjanna KM, Shivakumar B, Pramodkumar TM. *Arch. Appl. Sci. Res.*, **2009**, 1, *230-253*
[48] Filisetti-Cozzi TMCC, Carpita NC. *Anal. Biochem*., **1991**, 197, 157–162.
[49] Ghildyal P, Gronhaug TE, Rusten A, Skogsrud M. *J. of Pharmacognosy and phytotherapy* **2010,** 2, 76-85
[50] Romeo A, Toffano G, Callegaro L. New use of acidic polysaccharide esters as anti-ulcer agents. **1993**, no. WO1993005792A1
[51] Byeon, SE, Lee J, Kim, J-H, Yang, WS, Kwak, YS, Kim, SY, Choung, ES, Rhee, MH, Cho JY. *Mediators of Inflammation*, **2012**, 2012, 732860.
[52] Jong-Min P, Ki-Baik H, Sang-Oh K, Eun-Hee K. *J. of Cancer Prevention* **2013**, 18, 161-168
[53] Liu W, Wang H, Pang X, Yao W, Gao X . *Inter. J. Biol. Macromol.*, **2010**, 46, 451-457.
[54] Kalka-Moll WM, Tzianabos AO, Wang Y, Carey VJ, Finberg RW, Onderdonk AB, Kasper DL. *J. Immunol.*, **2000**, 164, 719-724;
[55] Yan J-K, Wang W-Q, Ma H-L and Wu J-Y. *Molecules* **2013**, 18, 167-177.
[56] Deux J-F, Anne M-P, Le Blanche A F, Feldman LJ, Sylvia C-J, Brée F, Boudghène F, Michel J-B, Letourneur D. *Thromb Vasc Biol.*, **2002**, 22, 1604-1609
[57] Hwang HJ, Kim, SW, Choi JW, Yun JW. *Enzyme and Microbial Technology*, **2003**, 33, 309–319.
[58] Kanmani P, Kumar RS, Yuvaraj N, Parri KA, Pattukumar V, Arul V. *Bioresource Technology*, **2011**, 102, 4827–4833.
[59] Sun RC, Fang JM, Goodwin A, Lawther JM, Bolton AJ. *Carbohydrate Polymers*, **1998**, 37, 351–359.
[60] Cheng A, Wan F, Jin Z, Wang J, Xu X. *J. of Ethnopharmacology*, **2008**, 118, 59–64.
[61] Shina E-J, Jeong J H, Chung Y H, Kim W-K, Ko K-H, Bach J-H, Hong J-S, Yoneda Y, Kim H-C. *Neurochem Int.*, **2013**, 59, 122–137.
[62] Yao M, Nguyen T-V, Pike CJ. *The J. of Neuroscience*, **2005**, 25, 1149 –1158.
[63] Singhal G, Jaehne EJ, Corrigan F, Toben C, Baune BT. *Front. Neurosci.*, **2014**, 8, 315.
[64] Minghetti L. *J. Neuropathol. Exp. Neurol.*, **2004**, 63, 901–910.
[65] Nivsarkar M, Aryamitra B, Harish P. *Pharmacological Reports*, **2008**, 60, 692–698
[66] Greig NH, Giacobini E, Lahiri DK. *Curr. Alzheimer Res*., **2007**, 4, 336-339.
[67] Pakaski M, Rakonczay Z, Kasa P. *Neurochem. Int*., **2001**, 38, 219-226.
[68] Oliva CA, Vargas JY, Inestrosa NC. *Front Cell Neurosci*, **2013**, 7, 224.
[69] Bakhle YS. *British Journal of Pharmacology*, **2001**, 134, 1137-1150
[70] Duh PD, Tu YY, Yen GC. *Lebensmittel-Wissenschaft and Technology*, **1999**, *32*, 269–*277*.
[71] Tsiapali E, Whaley S, Kalbfleisch J, Ensley HE, Browder IW, Williams DL. *Free Radical Biology and Medicine*, **2001**, 30, 393–402.
[72] Moure A, Dominguez H, Parajo JC. *Process Biochemistry*, **2006,** 41, 447–456.